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TITLE: **ANTI-LTNF FOR IN VITRO ASSAY
OF BIOLOGICAL TOXINS**

BACKGROUND OF THE INVENTION

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The invention relates to the introduction of a novel reagent for estimating biological toxins by in vitro test, as a replacement of currently used animal bioassay.

15   The direct testing of the toxins on animals poses ethical and moral questions. Therefore, there is great need for an alternative assay for biological toxins as a replacement for animal testing.

Biological toxins are grouped according to the source, such as:
20   animal, plant, unicellular or one celled algae, and bacteria. Toxins are diverse and range from well-defined single macromolecules (tetanus, diphtheria, and botulinum toxins) to mixtures of complex molecules such as: snake or scorpion venoms or simple chemical entities (digitoxin, colchicine, tricyclic anti-depressants).

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Mouse bioassay is the accepted and practiced method for assaying toxins, and is recommended by the Association of Official Analytical Chemists (AOAC). However, the mouse test for biological toxins is expensive and is disliked by animal activists and pressure is
30   mounting worldwide to eliminate live animal bioassays. Germany, Switzerland and Australia have already banned the use of mammals for venom lethality tests.

The mouse bioassay suffers from other shortcomings as well. Toxins not lethal to mouse will go undetected by mouse bioassay. Also, mouse bioassay collectively detects the lethal effects of toxin, whether it is a single toxin or a mixture of several toxins.

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Numerous investigators have developed immunological tests for assaying various toxins. Immunological tests are based on the principle of antigen-antibody reaction. Polyclonal antibodies are raised by immunizing animals: mouse, rabbit, rat, goat, etc. with a

10 toxin.

· Monoclonal antibodies are prepared in hybridoma cells. Spleen cells from an animal immunized with desired toxin are fused with corresponding species of myeloma cells. Both monoclonal and

15 polyclonal antibodies produced are specific to the toxin (antigen used for immunization), and react with specific toxin.

The most frequently used in vitro immunological test is Enzyme Linked Immunosorbent Assay (ELISA). Biological toxins are assayed

20 by several types of ELISA tests using specific antibodies to the desired toxin. ELISA tests can be carried out with numerous variations, although the most common format for detecting toxin from serum samples is the antigen-capturing method also known as double-sandwich method.

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Li and Ownby reported the development of ELISA test for identification of venoms from snakes in the Agkistrodon genus. Several investigators have reported the development of ELISA for different toxins such as ricin and botulinum toxins; types A, B, and E

30 in inoculated food samples. Morton and Tindall compared three tests; HPLC fluorescent method and two monoclonal antibody test kits for the determination of okadaic acid content of dinoflagellate cells and the results were not consistent. Since outbreaks of diuretic shellfish poisoning (DSP) may be caused by okadaic acid, methylokadaic acid,

35 or a combination of these toxins, they concluded that both kinds of ELISA kits may underestimate total toxin effect in toxic shellfish.

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All these methods suffer disadvantages, which have prevented their wide spread implementation particularly under the regulatory requirements. The mouse bioassay detects a wide range of known and presumably unknown toxins. Therefore, it is unlikely that it will be abandoned completely in favor of existing bioassays, until an alternative is found which is similarly responsive.

What is needed is a reagent that can be used in a common protocol for an in vitro assay test for biological toxins.

What is further needed is an in vitro test that can recognize all types of toxins, as a replacement of animal use.

OBJECTS OF THE INVENTION

An object of the present invention is to provide a novel reagent, specifically, Anti Lethal Toxin Neutralizing Factor (anti-LTNF), having a property to bind to all types of toxins derived from animal, plant and bacteria and can be assayed by immunological in vitro test, specifically, ELISA.

Another object of the invention is to provide a method to use Anti-LTNF having such a unique property to recognize all types of toxins in vitro test with sensitivity greater than in vivo mouse test.

Another object of the invention is to provide anti-LTNFs (made versus natural and synthetic LTNF) for use in a simple, cheap, ethically accepted in vitro alternative as a replacement for the currently used animal bioassay.

Another object of the invention is to provide a method of identifying toxins by an ELISA sandwich utilizing anti-LTNF as primary antibody and a set of secondary antibodies specific to the known toxins selected for the test.

SUMMARY OF THE INVENTION

In one embodiment of the invention, there is provided the novel composition of matter, anti-LTNF. Anti-LTNF is the antibody produced biologically in response to natural Lethal Toxin neutralizing Factor (LTNF-n) or LTNF-s the synthetic active domain from LTNF-n. These materials are generically referred to herein as LTNFs. LTNF-n is a protein isolated from opossum serum having a molecular weight of 68 kDa and the amino acid sequence from the N-terminal of Leu Lys Ala Met Asp Pro Thr Pro Pro Leu Trp Ile Lys Thr Glu. LTNF-s is a synthetic fragment of LTNF-n consisting of the portion of the N-terminal sequence of LTNF-n. A highly desirable active species of anti-LTNF is produced biologically in response to an LTNF-s having the first ten amino acids of the above sequence.

In another embodiment of the invention, an unknown toxin in fluid state is brought together with an anti-LTNF. The resulting immunological reaction indicates the presence of toxin in the fluid. The immunological reaction can determine the potency of the toxin.

Because anti-LTNF immunologically binds to all types of toxins, it can be used in vitro to test toxicity or to test for the presence of toxins as an alternative to animal testing. It can also be used for the identification of toxins by reacting with specific antitoxins under investigation.

DETAILED DESCRIPTION OF THE INVENTION

Anti-LTNF is the antibody made versus LTNF-n and anti- LTNF-s is made versus LTNF-s.

As used herein, LTNF-n refers to natural LTNF isolated from opossum serum having a molecular weight of about 68 kDa. The amino acid (AA) sequence from the N-terminal for the first fifteen amino acids of LTNF-n is:

Leu Lys Ala Met Asp Pro Thr Pro Pro Leu Trp Ile Lys Thr Glu.

For additional details, see US Patent 5744449, the disclosure of which is incorporated by reference.

5   The synthetic LTNF comprising of 15 amino acids was identified as the active domain of the LTNF-n.

Synthetic LTNF or LTNF-s refers to a synthetic peptide having a molecular weight of less than 2,500 and at least a portion of the sequence set forth above. Preferably, LTNF-s has at least a 3-amino

10   acid portion of the sequence, more preferably, at least a 5 amino acid portion of the sequence, and most preferably, at least a 7 amino acid portion of the sequence. Specific examples of LTNF-s include LTNF-15, LTNF-10 and LTNF-5. LTNF-15 is the 15 amino acid peptide having the first 15 amino acids of the above sequence and a molecular

15   weight of 1,989 daltons. LTNF-10 is the 10 amino acid peptide having the first ten amino acids of above sequence with molecular weight of 1242 daltons. LTNF-5 is the 5 amino acid peptide having the first five amino acids of the above sequence with molecular weight of 647 daltons.

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PRODUCTION OF ANTI-LTNF

Antibodies in general, are produced in animals by injecting the desired protein, which makes specific circulating antibodies to the

25   protein. The serum of the immunized animal reacts immunologically to the protein, which was used for immunization. The presence of antibodies to the protein can be detected by various in vitro tests such as ELISA and immunoprecipitin test (IP).

30   LTNF-n and synthetic peptides consisting of fifteen, ten and five amino acids, designated as LTNF-15, LTNF-10 and LTNF-5 were used to produce antibodies by immunizing Balb/C mice. The sera of the immunized mice contained specific antibodies. In other words, mice immunized with LTNF-n produced antibodies specific to LTNF-n, anti

35   LTNF-n is formed. Likewise, mice immunized with synthetic peptides

LTNF-15, LTNF-10 and LTNF-5 produced anti-LTNF-15, anti-LTNF-10 and anti-LTNF-5 respectively.

5   (1) Polyvalent anti-LTNFs can be made in animals, mice, rabbits, goats etc. (2) monoclonal anti-LTNFs can be made in fused hybridoma cells secreting antibodies (3) or anti-LTNFs can be made in ascitic cavities of mice by injecting hybridoma antibody secreting cells.

10 There is a perception that small synthetic peptides do not generate antibodies on injection into animals. However, synthetic peptide can generate antibodies if it is tagged with a complete protein, before injecting to the animal. Landsteiner coined the term hapten for a low molecular weight, chemically defined compound which could induce antibody formation only when coupled to a larger carrier protein
15 molecule, before injecting. Thereby, the injected animal makes antibodies to both the hapten and the carrier protein.

Synthetic chemically defined LTNFs comprising of fifteen, ten and five amino acids can be considered as haptens and therefore, theoretically
20 should not induce antibodies if injected without carrier protein. However, surprisingly, synthetic LTNF consisting of fifteen amino acids was able to induce antibodies on injecting in mice. Encouraged by these results, mice were injected with synthetic LTNFs consisting of ten and five amino acids to induce antibodies, in a similar protocol.
25 Each synthetic LTNF consisting of fifteen, ten and five amino acids was able to induce specific antibodies as assayed by ELISA and IP.

SEPARATION OF ANTI-LTNF IgG

30 **Purification of IgG from Mouse anti-LTNFs:** Purification of immunoglobulin (IgG) can be accomplished by various methods and one of them is by high pressure liquid chromatography (HPLC). Anti sera were fractionated on HPLC from Tosoh Co. (Japan) using ionic exchange column from Polymer Laboratories (UK) and Trizma-HCl
35 gradient buffer pH 7.4. For a run, 20 mg of serum protein containing desired antibody was loaded for fractionation. The IgG fraction was

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collected dialyzed and concentrated using apparatus from Spectrum Co. The concentrated fraction of IgG was refractionated on HPLC under identical conditions to obtain homogeneous preparation of IgG.

USES OF ANTI-LTNFs

Anti-LTNFs can be used to assay the toxicity of venom or venom toxins.

Anti-LTNFs can be used to assay the toxicity of toxins from sources other than venoms.

Anti-LTNFs can be used to detect the presence of multiple unrelated toxins.

Anti-LTNFs can be used to detect the presence of toxins in foods or biological fluids such as blood serum, saliva, urine, milk, tears etc.

Anti-LTNFs can be used to determine the potency of antivenoms and antitoxins.

The unique property of anti-LTNF to bind to all tested types of toxins immunologically in a fashion comparable to animal bioassay, is the theme of this patent. The protein LTNF-n and the synthetic peptides LTNF-15, LTNF-10 and LTNF-5 do not react immunologically with toxins by ELISA or IP tests. However, the anti-LTNFn, anti LTNF-15, anti LTNF-10 and anti LTNF-5 react immunologically with all tested toxins, as revealed by ELISA and IP tests. Moreover, the binding affinity of anti-LTNFn and the anti-synthetic LTNF-15, LTNF-10 and LTNF-5 roughly proportional to the lethal dose of the toxin under testing.

Using the novel reagent anti-LTNF, ELISA tests can be performed in several different ways: Furthermore, anti-LTNFs can be used in crude form as anti serum, or it's purified IgG. However, to avoid background purified, it is ideal to use IgG for ELISA tests.

ELISA tests for assaying toxins can be performed by using different concentrations of toxins and reacting with constant amount of purified IgG from anti-LTNF.

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ELISA Method in Antigen Capture Format: ELISA can be done in antigen capture format (also called double sandwich) where anti-LTNF IgG from one species (mouse) is used to capture the toxin and anti-LTNF from another species (rabbit) is used to recognize the toxin. For

10   commercialization, two types of antibodies either polyclonal or monoclonal, to LTNFs, natural, or synthetic, should be used. One for capturing the toxin and other for conjugating with alkaline phosphotase or peroxidase to recognize the toxin as in the antigen capture format.

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Toxin detection from foods, sera or other body fluids must be done by antigen capture format. The second antibody can be conjugated directly to alkaline phosphatase or horse radish peroxidase to eliminate one step of washing the plate and to save one-hour

20   incubation period. IgGs from all four anti-LTNFs to: LTNF-n, LTNF-15, LTNF-10 and LTNF-5 were tested in ELISA immunological tests and were found equally good.

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Example I -- Production of polyclonal antibodies

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Production of Polyclonal Antibodies to LTNFs (LTNF-15, LTNF-10 and LTNF-5): Adult Balb/C mice were used for immunization. The mice were used in compliance with the U.S. Public Health Service policy on humane care and use of animals. For the first injection

10 each type of LTNF was mixed with equal amount of Freund's complete adjuvant and for the subsequent doses, the antigens were mixed with incomplete Freund's adjuvant. The mice were injected intra muscularly with 100 µg/mouse, four to six times, two weeks apart. At the end of the immunization period, mice were bled through

15 ophthalmic vein and sera were separated.

Enzyme-Linked Immunosorbent Assay (ELISA) for Cross Reactivity of Anti-LTNFs:
ELISA tests were performed in 96 well microtiter plate. The plate was

20 coated with one concentration of antigen. The wells of the plate were filled with 10 µg/ml in 0.05 M phosphate buffered saline (PBS) pH 7.3 and 100 µl/well, for each type of LTNFs such as natural LTNF (LTNF-n), synthetic peptide consisting of 15 amino acids (LTNF-15), consisting of 10 amino acids (LTNF-10) and consisting of 5 amino

25 acids (LTNF-5). The plate was incubated for overnight at room temperature. After 18 to 24 hours the plate was washed three times (3X) with PBS and the plate was blocked with 0.25 ml/well of 3% Teleostean gelatin from cold water fish (Sigma) for 1/2 hour at RT.

30 Mouse anti-LTNFn, anti-LTNF-15, anti-LTNF-10 and anti-LTNF-5 diluted three fold were added to the coated plate and the plate was incubated at 37°C for 1 to 2 hours. The plate was washed 3X with PBS and horseradish peroxidase conjugated with mouse IgG made in goat (Sigma) was added and incubated for 1 hour at 37°C.

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After which the plate was washed 3X with PBS and reacted with O Phenylenediamine Dihydrochloride (OPD) for color development. The test was read after 1/2 hour visually or preferably on ELISA plate reader. The results are presented in table 1.

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Immunoprecipitation Test (IP):

IP was carried out as described by Ouchterlony. Agarose in 1-% concentration was dissolved by heating in normal saline and 7 ml of it was placed in 35-mm diameter petri dish. After the hardening of

10   agarose, five wells were punched, one in the center and four peripheral wells, 1.0 cm apart from the central well. The central well was filled with one type of undiluted anti serum and the peripheral with four LTNFs having concentration 5 mg/ml. The wells were filled several times 50 µl each time, over the period of 72 hours. The results

15   are presented in table 1.

Table 1: Immunological Cross Reactivity of
Natural and Synthetic LTNFs by ELISA and by IP.

	ELISA titer				Immuno. Precipitation			
Antigen	Anti LTNF	Anti LT15	Anti LT10	Anti LT5	Anti LTNF	Anti LT15	Anti LT10	Anti LT5
Nat- LTNF	24300	600	2700	900	+	+	+	+
Syn- LTNF-15	8100	2700	2700	900	+	+	+	-
Syn- LTNF-10	8100	8100	8100	900	-	-	+	-
Syn- LTNF-5	8100	900	2700	600	-	-	+	-

20   The results of table 1 show that synthetic peptides consisting of as small as five amino acids are capable of producing antibodies in mice. The antibodies versus LTNF-15, LTNF-10 and LTNF-5 cross react with each other and with the natural LTNF-n by ELISA and by IP. Among

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the three, anti-LTNF-10 proves to be better than anti-LTNF-15 and anti-LTNF-5.

5   It appears that the active domain of LTNF-n resides in ten amino acids. Therefore, LT-10 produced antibodies having high potency immunologically. LT-15 is may be too long having five amino acids not necessary for the active domain and LT-5 is too short of five amino acids, to generate antibodies having similar immunological potency.

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Example II -- Immunological reaction of Anti-LTNF with Venom Toxin

15   **Immunological binding of anti-LTNF to venoms by ELISA:** The lethal dose was determined by injecting intraperitoneally 0.1 ml of venom in various concentrations in 20g ICR mice. ELISA test was carried in 96 well microplate. The wells of the microplate were coated with 0.1 ml of various concentrations of venom as antigen starting
20   from 100 μg to 0.000564 (564 nanogram) diluted threefold in 0.05 M phosphate buffer saline pH 7.4 (PBS) and incubated for overnight at room temperature. After 18 to 24 hours the plate was washed three times (3X) with PBS and the plate was blocked with 0.25 ml/well of 3% Teleostean gelatin from cold water fish (Sigma) for 1/2 hour at RT.
25   The plate was washed 3X with PBS and 0.1 ml/well of 10 μg/ml purified mouse anti-LTNF IgG was added. The plate was incubated at 37°C for 1 to 2 hours.   And then, the plate was washed 3X with PBS and horseradish peroxidase conjugated with mouse IgG made in goat was added and incubated for 1 hour at 37°C. After which the plate
30   was washed 3X with PBS and reacted with O Phenylenediamine Dihydrochloride (OPD) for color development. The test was read after 1/2 hour visually or preferably on ELISA plate reader. The results are shown in table 2.

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Table 2: Immunological binding of anti-LTNF-n to venoms.

Venom	Lethal dose μg	ELISA dose μg	Ratio L/E
Crotalus atrox (Rattlesnake)	300	3.7	81
Naja.n. kaouthia (Thailand cobra)	35	0.1	350
Vipera russelli (Common viper)	90	0.4	225
Oxyuranus scutellatus (Australian taipan)	3.5	0.02	175
Androctonus australis (Scorpion)	15	0.2	75
Astrotia stokesii (Sea snake)	4.0	0.03	133

The results of table 2 show that the toxicity of venoms was roughly proportional to the ELISA binding or ELISA titer. The mouse lethal

10   dose for C. atrox venom was 300 μg and ELISA was 3.7 μg. Mouse lethal dose for sea snake Astrotia stokesii was 4 μg and its ELISA was 0.03 μg.

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Example III -- Toxicity is proportional to binding affinity with toxins as well

Mouse Lethality of Toxins and Binding Affinity to Anti-LTNF IgG

5 **by ELISA:** The lethal dose was determined by injecting intraperitoneally 0.1 ml of toxin at various concentrations in 20 g ICR mice. ELISA test was performed as described above using three fold concentrations of toxin and 10 µg/ml anti-LTNF IgG. Results are shown in table 3.

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Table 3: Mouse lethality of toxins in micro grams and binding affinity to anti-LTNF IgG by ELISA

Toxin	Source	Lethal dose µg	ELISA dose µg	Ratio L/E
BoTx	*Clostridium botulinum*	1	0.04	25
Cholera	*Cholerae vibrio*	ND*	0.11	
Cobratoxin	*Naja n. kaouthia*	3	0.2	15
Crotoxin	*Crotalus d. terrificus*	5	1.1	4.5
Holothurin	*Actinopyga agrassizi*	200	1.8	111
Ricin	*Ricinus communis*	2	0.04	50
Taipoxin	*Oxyuranus scutellatus*	5	1.1	4.5
Toxic shock	*Staphylococeus aureus*	ND*	0.33	

* ND = not determined because these toxins are not lethal to adult mice.

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The results of table 3 show that lethality of toxins is roughly proportional to the ELISA binding or ELISA titer. Mouse lethal dose for BoTx (botulinum toxin) was 1 µg and ELISA detection level was found to be 0.04 µg, whereas, for Holothurin mouse lethal dose was 20 200 µg and its detection level by anti-LTNF was 1.8 µg. Lower the ELISA binding means higher the detection level.

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Example IV -- Multiple Toxins can be detected

Anti-LTNFs Detect the Presence of Multiple Unrelated Toxins:
Equal volumes of ricin at 10 µg/ml was mixed with equal
5   concentration of different toxins; BoTx, Cholera toxin and
Staphylococcus aureus toxin.   ELISA tests were carried using
individual toxin and ricin plus other toxin as antigens.   The toxins
individually and the mixtures were diluted three fold. The binding was
assayed by IgGs from anti-ricin and anti-LTNF.   The results are shown
10   in table 4.

Table 4: Anti-LTNFs detect presence of several unrelated toxins

Toxin	Binding affinity, µg	
	Anti-Ricin	Anti-LTNF
Ricin	330	400
Ricin+BoTx	330	370
BoTx	0	37
Ricin+Cholera	330	110
Cholera	0	37
Ricin+Sta. aureus	330	12.3
Toxic shock	0	45

The results clearly show that anti-ricin detects only ricin by classical
15   specific antigen-antibody reaction and non-of the other toxins mixed
with it.   However, anti-LTNF detects the presence of ricin as well as
other toxins; BoTx, Cholera and Sta. aureus. Thus, anti-LTNF has the
unique property of binding with all tested types of unrelated biological
toxins, individually or in mixture.

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Example V -- Toxins in Food or animal body fluids can b detected

ELISA Test for Detection of Toxins from Foods by Anti-LTNF:
5   Human serum, human urine, chicken broth and dairy milk were spiked with 10 μ/ml of different toxins; BoTx, Ricin, Cholera and Sta. aureus. ELISA test was carried as described previously, using anti-LTNF IgG. The results are seen in table 5.

10   **Table 5: Anti-LTNF detects toxins from foods and body fluids**

Diluent	Detection Level in nanogram			
	BoTx	Ricin	Cholera	Sta. Aureus
PBS	333	333	111	333
Serum	11	11	11	11
Milk	220	110	111	111
Broth	330	110	111	111
Urine	330	220	12.3	37

BoTx: Botulium toxin from Clostridium botulinum.
Ricin: Plant toxin from Castor seeds.
Cholera: Toxin from Vibrio cholerae.
Sta. aureus: Toxin from Staphylococcus aureus.

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The detection levels of BoTx, ricin, cholera and toxic shock was the lowest in human serum -- down to 11 ng (This corresponds to the highest sensitivity). Detection levels in milk were higher than observed in broth and urine (except for BoTx). The detection levels for
20   cholera and Sta. aureus were lower in urine than in milk and broth.

Note: The concentrations of serum, milk, broth and urine diluted threefold in PBS for the obtained detection levels without added toxins were ELISA negative. In other words the positive reaction was not due
25   to the protein present in diluent but due to the presence of added toxin. Thus, anti-LTNFs are useful for in vitro assaying various types of food poisoning toxins from liquid and solid foods.

Example VI -- Antivenom and Antitoxin potency can be determined.

Neutralization assay of Anti-venoms using anti-LTNF: Currently,
5   potency of antivenoms and anti toxins is measured by neutralization
tests in mice. Neutralization test requires numerous mice because
this test requires a determination of the LD_{50} of venom alone and for
the venom neutralized by the antiserum. At least four different
concentrations of venom alone and a similar number or more for the
10   venom to be neutralized by the antivenom must be used. In order to
achieve statistical significance five to six mice are used per
concentration. Thus, to determine the potency of a single antivenom
fifty mice are required. (This is undesirable.)

15   The protein concentration of each venom was adjusted to 200 µg/ml.
Each venom was mixed with equal amount of normal rabbit serum
and homologous respective rabbit anti serum. The mixtures were
incubated at 37°C for one hour. The mixtures of venom with normal
serum and specific anti serum were considered as antigens for ELISA.
20   ELISA test was performed as described above. The results of detection
levels of venom toxins in the mixtures are shown in table 6.

Table 6: Neutralization assay of venoms using anti-LTNF

Venom	Binding to anti-LTNF µg	Neutralization Index
Crotalus atrox + NS	3.7	
Crotalus atrox + anti serum	1.2	2.5
Naja. n. kaouthia + NS	1.2	
Naja. n. kaouthia + anti serum	0.04	1.16
V. russelli + NS	3.7	
V. russelli + anti serum	0.8	2.9
O. scutellatus + NS	0.8	
O. scutellatus + anti serum	0.2	0.6

NS= normal rabbit serum
25   Homologous rabbit anti serum specific to each venom